

## THREE COMPONENT CHIMERIC ANTISENSE OLIGONUCLEOTIDES

### 1. FIELD OF THE INVENTION

This invention relates to antisense oligonucleotides  
5 that target mRNAs in cells as substrates for the cellular  
enzyme RNase H and thereby cause specific degradation of the  
targeted mRNA. The oligonucleotides have three components: a  
RNase H activating region, a complementarity region and 3'  
and 5' ends. The invention optimizes each of the components  
10 to resist intracellular nucleases, to increase hybridization  
to target mRNA, to specifically inactivate target mRNA in  
cells, and to decrease cytotoxicity.

### 2. BACKGROUND TO THE INVENTION

15 Antisense polynucleotides are useful for  
specifically inhibiting unwanted gene expression in mammalian  
cells. They can be used to hybridize to and inhibit the  
function of an RNA, typically a messenger RNA, by activating  
RNase H.

20 The use of antisense oligonucleotides has emerged as a  
powerful new approach for the treatment of certain diseases.  
The preponderance of the work to date has focused on the use  
of antisense oligonucleotides as antiviral agents or as  
anticancer agents (Wickstrom, E., Ed., Prospects for  
25 Antisense Nucleic Acid Therapy of Cancer and AIDS, New York:  
Wiley-Liss, 1991; Crooke, S.T. and Lebleu, B., Eds.,  
Antisense Research and Applications, Boca Raton: CRC Press,  
1993, pp. 154-182; Baserga, R. and Denhardt, D.T., 1992,  
Antisense Strategies, New York: The New York Academy of  
30 Sciences, Vol. 660; Murray, J.A.H., Ed., Antisense RNA and  
DNA, New York: Wiley-Liss, 1993).

There have been numerous disclosures of the use of  
antisense oligonucleotides as antiviral agents. For example,  
Agrawal et al. report phosphoramidate and phosphorothioate  
35 oligonucleotides as antisense inhibitors of HIV. Agrawal et  
al., Proc. Natl. Acad. Sci. USA 85, 7079-7083 (1988).  
Zamecnik et al. disclose antisense oligonucleotides as

inhibitors of Rous sarcoma virus replication in chicken fibroblasts. Zamecnik et al., Proc. Natl. Acad. Sci. USA 83, 4143-4146 (1986).

The principal mechanism by which antisense  
5 oligonucleotides affect the level of the target RNA is by activation of RNase H, which cleaves the RNA strand of DNA/RNA hybrids. Both phosphodiester and phosphorothioate-linked DNA activates endogenous RNase H, thereby cleaving the targeted RNA (Agrawal, S., et al., Proc. Natl. Acad. Sci. USA  
10 87, 1101-5 (1990); Woolf, T.M., et al., Nucleic Acids Res. 18, 1763-9 (1990)). However, phosphodiester-linked DNA is rapidly degraded by cellular nucleases and, with the exception of the phosphorothioate-linked DNA, nuclease resistant, non-naturally occurring DNA derivatives do not  
15 activate RNase H when hybridized to RNA. While phosphorothioate DNA has the advantage of activating RNase H, phosphorothioate-linked DNA has non-specific cytotoxic effects and also has reduced affinity for RNA (Stein, C.A., et al., Aids Res Hum Retroviruses 5, 639-46 (1989); Woolf,  
20 T.M., et al., Nucleic Acids Res. 18, 1763-9 (1990); Kawasaki, A.M., et al., J. Med. Chem. 36, 831-41 (1993)).

Chimeric antisense oligos that have a short stretch of phosphorothioate DNA (3-9 bases) have been used to obtain RNase H-mediated cleavage of the target RNA (Dagle, J.M., et  
25 al., Nucleic Acids Res. 18, 4751-7 (1990); Agrawal, S., et al., Proc. Natl. Acad. Sci. USA 87, 1401-5 (1990); Monia, B.P. et al., 1993, J. Biol. Chem. 268, 14514) A minimum of 3 DNA bases is required for activation of bacterial RNase H (Futdon, P.J., et al., Nucleic Acids Res. 17, 9193-9204;  
30 Quartin, R.S., et al., Nucleic Acids Res. 17, 7235-7262) and a minimum of 5 bases is required for mammalian bacterial RNase H activation (Monia, B.P., et al., J. Biol. Chem. 268, 14514-14522 (1993)). In these chimeric oligonucleotides there is a central region that forms a substrate for RNase H  
35 that is flanked by hybridizing "arms," comprised of modified nucleotides that do not form substrates for RNase H. Alternatively, extracellular tests using a RNase H-containing

HeLa cell extract have been reported wherein the RNase H activating region was placed on the 5' or 3' side of the oligomer. Specifically these tests reported that a 5' or 3' terminal RNase H activating region composed of phosphodiester 2'-deoxynucleotides joined to a methylphosphonate-linked complementarity region was fully active, but that a 5' terminal RNase H-activating region composed of phosphorothioate 2'-deoxynucleotides joined to a methylphosphonate-linked complementarity region was only partially active. See Col 10, U.S. Pat. No. 5,220,007 to T. Pederson et al.

2'-O-Methyl or 2'-fluoro modified nucleotides have been used for the hybridizing arms of chimeric oligos. Inoue, H., et al., 1987, Nucleic Acids Res. 15, 6131-48. The 2'-O-Methyl group increases the affinity of the oligomer for the targeted RNA and increases the activity of the oligomer in cell culture. However, 2'-O-Methyl bases with phosphodiester linkages are degraded by exonucleases and so are not suitable for use in cell or therapeutic applications of antisense. Shibahara, S., et al., 1989, Nucleic Acids Res. 17, 239-52. Phosphorothioate 2'-O-Methyl nucleotides are resistant to nucleases as shown in the uniformly phosphorothioate modified oligos described by Monia B.P., et al., 1993, J. Biol. Chem. 268, 14514-14522 and terminal phosphorothioate substituted, 2'-O-Methylribo-oligonucleotides, Shibahara, S., et al., 1989, Nucleic Acid Res. 17, 239-252. However, fully phosphorothioate substituted oligomers may cause non-specific effects including cell toxicity. Stein, C.A., et al., 1989, Aids Res. Hum. Retrov. 5, 639-646; Woolf, T.M., et al., 1990, Nucleic Acids Res. 18, 1763-69; Wagner, R.W., 1995, Antisense Res. Dev. 5, 113-115; Krieg, A.M., & Stein, C.A., 1995, Antisense Res. Dev. 5, 241. The effects of 2'-Fluoro-oligonucleotides on bacterial RNase H are discussed in Crooke, S.T. et al., 1995, Bioch. J. 312, 599-608 and Iwai, S. et al., 1995, FEBS Lett (Neth.) 368, 315-20.

Several other chemistries have been used to make the "arms" or regions of a chimeric oligomer that are not

substrates for RNase H. The first chimeric oligomers used methylphosphonate or phosphoramidate linkages in the arms (Dagle, J.M., Walder, J.A. & Weeks, K.L., Nucleic Acids Res. 18, 1751-7 (1990); Agrawal, S., et al., Proc. Natl. Acad. Sci. USA 87, 1401-5 (1990). While these compounds functioned well in buffer systems and *Xenopus* oocytes, the arms decreased the hybrid affinity. This decrease in affinity dramatically reduces the activity of oligomers in mammalian cell culture.

10 A number of studies have been reported for the synthesis of ethylated and methylated phosphotriester oligonucleotides and their physico-chemical and biochemical evaluation. Dinucleotides with methyl and ethyl triesters were shown to possess greater affinity towards polynucleotides possessing  
15 complementary sequences (Miller, P.S., et al., J. Am. Chem. Soc. 93, 6657, (1971)). However, a few years ago, another group reported lack of, or poor binding affinity of heptethyl ester of oligothymidine with complementary polynucleotides (Pless, R.C., and Ts'0, P.O.P., Biochemistry 16, 1239-1250  
20 (1977)). Phosphate methylated (P-methoxy) oligonucleotides were synthesized and found to possess resistance towards endonuclease digestion (Gallo, K.L., et al. Nucl. Acid Res. 18, 7405 (1986)). A P-methoxy 18-mer oligonucleotide was shown to have high T<sub>m</sub> value in duplexes with natural DNA and  
25 blocked to the DNA replication process at room temperature (Moody, H.M., et al., Nucl. Acid Res. 17, 4769-4782 (1989)). Moody et al. stated that phosphate ethylated (P-ethoxy) oligonucleotides would have poor antisense properties. P-methoxy dimers of DNA bases were synthesized using transient  
30 protecting group of FMOC for the exocyclic amino groups (Koole, L.H., et al., J. Org. Chem. 54, 1657-1664 (1989)).

Synthesis and physico-chemical properties of partial P-methoxy oligodeoxyribonucleotides were determined. Only the thymidine and cytidine oligomers with methyl phosphotriester  
35 could be prepared satisfactorily due to difficulty in maintaining methyl triester intact. Furthermore, the methyl group was found to have destabilizing effect on the

hybridization properties of the modified oligomers with its complementary sequence by comparison with unmodified parent oligodeoxynucleotide (Vinogradov, S., Asseline, U., Thoung, N.T., Tet. Let. 34, 5899-5902 (1993)).

5 Other reports have suggested that P-methoxy oligonucleotides are preferable to P-ethoxy as antisense oligonucleotides because of p-methoxy oligonucleotides showed stronger hybridization than methyl phosphonate or P-ethoxy oligonucleotides (van Genderen, M.H.P., et al., Kon. Ned. Akad. van Wetensch. B90, 155-159 (1987); van Genderen, M.H.P., et al., Trav. Chim. Pays Bas 108, 28-35 (1989)). P-ethoxy oligonucleotides were reported by van Genderen et al. to hybridize poorly to DNA, for which reason they were regarded unfavorably as antisense oligonucleotides (Moody, H.M., et al., Nucl. Acid Res. 17, 4769-4782 (1989)).

P-isopropoxyphosphoramidites have been synthesized from several nucleosides (Stec, W.J., et al., Tet. Let. 26, 2191-2194 (1985)), and a few short oligonucleotides containing P-isopropoxy phosphotriesters were synthesized, and hybridization studies were carried out.

United States Patent No. 5,525,719 to Srivastava, S., and Raza, S.K., June 11, 1996, suggests antisense oligonucleotides consisting of 2'-O-Methyl nucleotides linked by phosphodiester and/or P-ethoxy or P-methoxy, phosphotriester moieties.

Thus, currently there are no nucleic acid chemistries nor any chimeras that have been developed that optimally achieve all the features that are needed to provide an effective antisense oligonucleotide i.e. low toxicity, high specificity, nuclease resistance, ease of synthesis, RNase H compatibility.

### 3. SUMMARY OF THE INVENTION

The invention provides a class of oligonucleotide that is optimized to target a specific RNA for RNase H degradation and to be itself resistant to degradation within in plasma and within eukaryotic, especially mammalian cells. The

oligonucleotides of the invention contain no naturally occurring 5'→3'-linked nucleotides. Rather, the invention provides oligonucleotides having two types of nucleotides: 2'-deoxyphosphorothioate, which activate RNase H, and 2'-modified nucleotides, which do not. The linkages between the 2'-modified nucleotides can be phosphodiester, phosphorothioate or P-ethoxyphosphodiester. Activation of RNase H is accomplished by a contiguous, RNase H-activating region, which contains between three and five 2'-deoxyphosphorothioate nucleotides to activate bacterial RNase H and between five and ten 2'-deoxyphosphorothioate nucleotides to activate eukaryotic and, particularly, mammalian RNase H. Protection from degradation is accomplished by making the 5' and 3' terminal bases highly nuclease resistant and, optionally, by placing a 3' terminal blocking group.

In a preferred embodiment the RNase H activating region, which is composed of highly nuclease resistant phosphorothioate nucleotides is placed at the 5' end of the oligonucleotide.

#### 4. DETAILED DESCRIPTION OF THE INVENTION

##### 4.1 THE STRUCTURE OF THE OLIGONUCLEOTIDES

An oligonucleotide of the invention is comprised of a 3'-terminal 5'→3'-linked nucleoside and from 11 to 59 5'→3' linked nucleotides, which nucleotides can be 2'-deoxynucleotides or 2'-modified nucleotides, modified to enhance the hybridization of the oligonucleotide to the target mRNA, such as 2'-fluoro, 2'-methoxy, 2'-ethoxy, 2'-methoxyethoxy, 2'-allyloxy ( $-\text{OCH}_2\text{CH}=\text{CH}_2$ ) nucleotides (hereinafter "2'-modified nucleotides"). The 3' terminal nucleoside can, optionally, be 2'-modified nucleoside. Those skilled in the art appreciate that the 3'-OH of the 3' terminal base can but need not be esterified to a phosphate or phosphate analog. The 3' terminal residue is referred to as a nucleoside even though it may be a nucleotide.

The internucleotide linkages of an oligonucleotide of the invention can be phosphodiester, phosphorothioate or P-ethoxyphosphodiester moieties. The oligonucleotide has a 3' terminus and a 5' terminus that are protected from nuclease attack. The 3' terminus is protected by having the 3' most 5'→3' linkage or linkages be a phosphorothioate or a P-alkyloxyphosphotriester linkage and/or by having a substituted 3' terminal hydroxyl, e.g., a 3'→3' linked nucleotide, wherein the alkyloxy radical is methoxy, ethoxy or isopropoxy and, preferably, ethoxy. Preferably two or three 3' terminal internucleotide linkages are phosphorothioate or a P-alkyloxyphosphotriester linkages. To reduce nuclease degradation, the 5' most 3'→5' linkage preferably should be a phosphorothioate linkage or P-alkyloxyphosphotriester linkage. Preferably, the two 5' most 3'→5' linkages should be phosphorothioate linkages or P-ethoxyphosphotriester linkages. Optionally, the 5'-terminal hydroxyl moiety can be esterified with a phosphorus containing moiety, e.g., phosphate, phosphorothioate or P-ethoxyphosphate, without limitation.

The 3' terminal 5'→3'-linked nucleoside has a 3'-OH that can be optionally substituted by a blocking moiety that prevents 3'-exonuclease degradation of the oligonucleotide. In one embodiment, the 3'-hydroxyl is esterified to a nucleotide through a 3'→3' internucleotide linkage. Optionally, the 3'→3' linked nucleotide at the 3' terminus can be linked by a phosphorothioate moiety.

In a preferred embodiment, the oligonucleotide contains, exclusive of an optional blocking nucleotide, between 15 and 50 bases and more preferably between 20 and 30 bases and in a most preferred embodiment the oligonucleotide is 25 bases in length. The oligonucleotide of the invention contains a single contiguous RNase H-activating region of between three to ten 2'-deoxyphosphorothioate nucleotides. The length of the RNase H activating region to activate bacterial RNase H is preferably between three and five nucleotides; to activate a eukaryotic RNase H it is between five and ten nucleotides.



The preferred length of the RNase H-activating region for the activation of mammalian RNase H is nine nucleotides.

All 5'→3' linked nucleotides of the oligonucleotide that are not a part of the RNase H-activating region are 2'-5 modified nucleotides, which contribute to the target binding and form the complementarity determining region. The complementarity region can be a contiguous region or can be divided by the RNase H-activating region. In the preferred embodiment the complementarity region is a contiguous region, 10 and more preferably is 3' to the RNase H-activating region.

In a preferred embodiment all bases except the from one to three 3' most nucleotides and nucleoside, the 5' terminal nucleotide and RNase H activating region nucleotides are phosphodiester linked. Large amounts of contiguous 15 phosphorothioate linkages are detrimental to the function of the oligonucleotides of the invention. Preferably, therefore, the oligonucleotides contain not more than ten contiguous phosphorothioate linkages.

#### 20 4.2 THE SYNTHESIS OF THE OLIGONUCLEOTIDES

The oligonucleotides of the invention can be synthesized by solid phase or liquid phase nucleotide synthesis, however, synthesis by solid phase techniques is preferred. Phosphodiester and phosphorothioate linked oligonucleotides 25 can be synthesized, using standard reagents and protocols, on an automated synthesizer utilizing methods that are well known in the art, such as, for example, those disclosed in Stec et al., J. Am. Chem. Soc. 106, 6077-6089 (1984); Stec et al., J. Org. Chem. 50(20), 3908-3913 (1985); Stec et al., J. Chromatog. 326, 263-280 (1985); LaPlanche et al., Nuc. Acid. Res. 14, 9081-9093 (1986); and Fasman, G.D., Practical Handbook of Biochemistry and Molecular Biology 1989, CRC Press, Boca Raton, Florida, herein incorporated by reference.

The synthesis of 2'-O-alkyl-oligoribonucleotides, where 35 the alkyl groups are methyl, butyl, allyl or 3,3-dimethylallyl is reviewed by Lamond, Biochem. Soc. Trans. 21, 1-8 (1993). Intermediates that are useful in the synthesis



of 2'-O-methyl oligoribonucleotides are described in U.S. Patents No. 5,013,830, No. 5,525,719 and No. 5,214,135, which are hereby incorporated by reference.

The synthesis of 2'-fluorophosphodiester and 2'-  
5 fluorophosphorothioate oligonucleotides can be performed according to teaching of Kawasaki, A.M., et al., 1993, J. Med. Chem. 36, 831-41 and WO 92/03568; the synthesis of P-alkyloxyphosphotriester-linked oligonucleotides and 2'-modified oligonucleotides can be performed according to U.S.  
10 Patent No. 5,525,719, each of which is incorporated herein by reference. The synthesis of phosphorothioate oligodeoxynucleotides is taught by U.S. Patent No. 5,276,019 and No. 5,264,423, which is hereby incorporated by reference. Synthesis of 2'-substituted oligonucleotides can be performed  
15 by variations on the techniques disclosed therein.

The synthesis of the oligonucleotides of the invention must be conducted with great attention to quality control. It is particularly important that the phosphorothioate linkages not be contaminated with phosphodiester linkages.  
20 It is advisable to pre-test the individual reagent lots to ascertain that high coupling efficiency can be obtained therewith and to exercise all possible precautions to maintain anhydrous conditions.

The quality of the synthesis of oligonucleotides can be  
25 verified by testing the oligonucleotides by capillary electrophoresis and denaturing strong anion HPLC (SAX-HPLC). The method of Bergot & Egan, 1992, J. Chrom. 599, 35-42 is suitable. SAX-HPLC is particularly useful to verify that the phosphorothioate nucleotides are completely thiolated, i.e.,  
30 are not contaminated by a small percentage of phosphodiesters.

The synthesis of oligonucleotides having both phosphodiester and phosphorothioate linkages is associated with a side reaction whereby the phosphorothioate linkages  
35 are oxidized by the standard I<sub>2</sub> treatments that are used to oxidize the cyanoethyl phosphoramidite. This problem can be minimized but not eliminated by reducing the concentration or

I<sub>2</sub> to as low as 0.001 M. Therefore, in a preferred embodiment, all phosphorothioates of the oligonucleotides of the invention are found at the 5'-end, so that no phosphorothioate bond is exposed to I<sub>2</sub>.

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#### 4.3 THE USES OF THE OLIGONUCLEOTIDES

The oligonucleotides of the invention can be used as antisense oligonucleotides in a variety of *in vitro* experimental situations to specifically degrade an mRNA of  
10 unknown function and thereby determine its physiologic function.

The oligonucleotides of the invention can be also used in clinical practice for any disease and against any target RNA for which antisense therapy is now known to be suitable  
15 or which is yet to be identified. Medical conditions for which antisense therapy is reported to be suitable includes Respiratory Syncytial Virus infection, WO 95/22553 by Kilkuskie, Influenza Virus infection, WO 94/23028, and malignancies, WO 94/08003. Further examples of clinical uses  
20 of antisense oligonucleotides are reviewed, in summary form, in Glaser, V., 1996, Genetic Engineering News 16, 1. Targets of antisense oligonucleotides under that are the subjects of clinical trials include protein kinase C $\alpha$ , ICAM-1, c-raf kinase, p53, c-myb and the bcr/abl fusion gene found in  
25 chronic myelogenous leukemia.

### 5. EXAMPLES

#### 5.1 EXPERIMENTAL CONDITIONS

The oligonucleotides of the invention are demonstrated  
30 by a test transient expression system which includes an mRNA encoding the luciferase protein that has been modified to include a test sequence derived from the ras gene. The specific antisense effects of an oligonucleotide can be measured by comparing the luciferase production of the test  
35 cells with the production of control cells having the same expression plasmid except for the absence of the ras-derived

sequence. The oligonucleotides of the invention tested have the sequence:

5'-TTGCCCACACCGACGGCGCCCA-3' (SEQ ID NO: 1)

The details of the assay are as follows:

- 5 Plasmid Constructs.** The plasmid used for the studies contained a portion of the ras gene sequence fused to luciferase (Monia, B.P., et al. J. Biol. Chem. 267, 19954-19962 (1992)). The control luciferase plasmids did not contain the ras target sequence.
- 10 Cell Culture Assay.** HeLa cells were grown to 40-90% confluence in DMEM/10% FBS, Supplemented with glutamine, penicillin and streptomycin on gelatin coated 24 well plates. The gelatin coating was necessary for cell to remain adherent during the transfections. Prior to transfection the cells
- 15** were washed twice with PBS (containing magnesium and calcium). LIPOFECTIN™ was mixed gently and 6.6μl was added for each milliliter of reduced serum medium (OPTI-MEM™, Gibco/BRL, Gaithersburg, MD). Oligomers were added from 50-100μM concentrated stock to make a master mixture. The Opti-
- 20** MEM/LIPOFECTIN/oligomer solution was added to the cells and incubated for 4 hours (≈0.5mls for one well of a 24 well plate).

- A target transfection mixture was prepared by first diluting 5μl of lipofectin per ml of OPTI-MEM and mixing.
- 25** Next 5μg of luciferase target and 5μg of CMV β-galactosidase were added per milliliter of OPTI-MEM/LIPOFECTIN™ mixture. The transfection mixture was mixed gently and allowed to complex for about 15 minutes. The master mixture reduced error by assuring that the control and experimental cells
- 30** received the exact same cationic lipid/plasmid complex. The concentration of oligonucleotide in the culture medium was between 200nM and 400nM in all experiments. The oligonucleotide containing media was removed from the cells and replaced with growth media and incubated for an
- 35** additional 9-18 hours. The cell were rinsed with calcium and magnesium free media and the media was removed. The plates

were frozen at -70 for >20 minutes and 100-300 $\mu$ l of reporter lysis buffer (Promega, Madison WI) was added.

The cells were put through 2 more freeze thaw cycles, to assure complete lysis. Luciferase assays were preformed according to the manufacture's instructions (Promega, Madison WI) and luminescence was detected with a 96 well luminometer (Packard, Meriden CT).  $\beta$ -galactosidase assays were preformed (Galacton Plus, Tropix) according to manufactures instructions and detected on the Packard luminometer.

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## 5.2 EXPERIMENTAL RESULTS

The results of luciferase assay are presented in Table I below. The results are reported as the percent specific inhibition which were calculated as

15  $100 \times (1 - (LUC_T/LUC_C)^{OLIGO} / (LUC_T/LUC_C)^{NO\ OLIGO})$ ; wherein  $LUC_T$  and  $LUC_C$  are the luciferase levels found in the cells transfected with luciferase plasmids containing and lacking the ras gene insert (SEQ ID NO: 1); and the superscripts "Oligo" and "No Oligo" refer to the presence and absence of antisense  
20 oligonucleotides.

TABLE 1

| Oligo                 | Formula     | Specific inhibition |
|-----------------------|-------------|---------------------|
| Controls ("C")        |             |                     |
| 25 C1                 | 25Mo        | 26%                 |
| C2                    | 25Ms        | 15%                 |
| C3                    | 9Ds16Mo     | 15%                 |
| C4                    | 9Do16MoInvT | 0%                  |
| 30 C5                 | 9Dp16MoInvT | 18%                 |
| C6                    | 9Dp13Mo3Ms  | 14%                 |
| Controls with all "S" |             |                     |
| 35 S1                 | 25Ds        | 93%                 |
| S2                    | 16Ms8DsD    | 100%                |
| S3                    | 8Ms9Ds7MsM  | 97%                 |

|    |       |                        |     |
|----|-------|------------------------|-----|
|    | S4    | 9Ds15MsM               | 95% |
|    |       | 9Ds at 3' end ("3'I")  |     |
| 5  | 3'I1  | InvTMs15Mo9DsInvT      | 59% |
|    | 3'I2  | 2Ms14Mo9DsInvT         | 57% |
|    | 3'I3  | 4Ms12Mo9DsInvT         | 65% |
|    |       | 9Ds in Middle ("MI")   |     |
| 10 | MI1   | 5Ms3Mo9Ds4Mo3MsM       | 64% |
|    | MI2   | 2Ms6Mo9Ds7 (MsMo) InvT | 71% |
|    | MI3   | 3Ms6Mo9Ds6MoMsInvT     | 87% |
|    |       | 9Ds at 5' end ("5'I")  |     |
| 15 | 5'I1  | 9Ds16MoInvT            | 83% |
|    | 5'I2  | 9Ds15MoMsInvT          | 85% |
|    | 5'I3  | 9Ds16MoBiotin          | 90% |
|    | 5'I4  | 9Ds16Mp                | 91% |
| 20 | 5'I5  | 9Ds14MoMpD             | 90% |
|    | 5'I6  | 9Ds13Mo2MpD            | 94% |
|    | 5'I7  | 9Ds12Mo3MpD            | 94% |
|    | 5'I8  | 9Ds14MoMsD             | 93% |
|    | 5'I9  | 9Ds13Mo2MsD            | 97% |
| 25 | 5'I10 | 9Ds12Mo3MsD            | 95% |

Key: M and D refer to 2'-O-methyl- and 2'-deoxy-ribonucleotides, respectively. The letters "o", "s" and "p" refer to phosphodiester, phosphorothioate diester, and P-ethoxy-phosphotriester linked nucleotides. "InvT" refers to a 3'→3' or 5'→5' linked thymidine at the 3' or 5' end, respectively.

30 Table I shows the results of control oligos C1-C6, all phosphorothioate oligos S1-S4, and oligos of the invention having the RNase activating region at the 3' end (3'I1-3'I3), in the middle (MI1-MI3) and at the 5' end (5'I1-5'I10).  
35 Control oligos C1, C2, C5 and C6 showed low levels of specific inhibition because these oligos lacked an RNase H activating region. Oligos C3 and C4 were inactive because

the 3' was unprotected and because native ssDNA was unstable, respectively. All phosphorothioate oligonucleotides (S1-S4) showed specific inhibitions that ranged between 93% and 100%, as did oligonucleotides 5'I6-5'I10, which have a 5'-located  
5 RNase H activating region and two or three 3' terminal 2'O-methyl modified P-ethoxy or phosphorothioate linked nucleotides (Mp and Ms, respectively). Lower levels of specific inhibition were observed when oligonucleotides with 3' and mid-located RNase H activating regions were employed  
10 or when suboptimal 3' protecting groups were present.

Although the oligonucleotides of the invention having 5' RNase activating regions achieved specific inhibitions which were comparable to that achieved by the uniform phosphorothioate oligonucleotides, the oligonucleotides of  
15 the invention were superior in that their use was associated with significantly less toxicity. Table II shows specific inhibition, the average metabolic activity as percent of no oligo control, as determined by MTS assay, and the percent viable cells, as determined by trypan blue exclusion for the  
20 conventional ("C"), all phosphorothioate ("S"), 3'I, MI and 5'I oligonucleotides, as well as for three species.

TABLE 2

| 25 | Oligo                   | % INH Luc | % of Control<br>Metabolic<br>Activity | % of Viable<br>Cells |
|----|-------------------------|-----------|---------------------------------------|----------------------|
|    | All "O" Oligos<br>C1-C6 | 15%       | 94%                                   | 76%                  |
|    | All "S" Oligos<br>S1-S4 | 96%       | 25%                                   | 21%                  |
| 30 | 3'I (1-4)               | 60%       | 70%                                   | 61%                  |
|    | MI (1-3)                | 74%       | 77%                                   | 67%                  |
|    | 5'I (1-10)              | 91%       | 71%                                   | 60%                  |

The best oligos on the chart have high percentage values in  
35 all columns.

The results demonstrated that the oligonucleotides of the invention achieve levels of specific inhibition more than



four times greater than conventional oligonucleotides while showing toxicity levels that were substantially less than the phosphorothioate oligonucleotides. The optimal group, 5'I, showed specific inhibition that was comparable to the  
5 phosphorothioate oligonucleotides.

### 5.3 THE EFFECT OF THE LOCATION OF THE RNASE H ACTIVATING REGION

The cause of lower specific activity observed for the  
10 3'I and MI type oligonucleotides was investigated. One possibility was that the oxidation steps using 0.02M I<sub>2</sub> cause the oxidation of the phosphorothioate linkages to phosphodiester, when phosphodiester linked nucleotides were added 5' to the phosphorothioate linkages. This was found to  
15 be the case. Comparison of oligonucleotides 9D<sub>s</sub>15D<sub>o</sub>D ("5'S") and 15D<sub>o</sub>9D<sub>s</sub>D ("3'S") oligonucleotides having the sequence of the test oligonucleotide by analytical HPLC analysis showed that about 85% of the 5'S oligonucleotides were fully  
20 thiolated, by contrast only 26% of the 3'S oligonucleotides were completely thiolated (36% were S-1, 24% S-2 and 14% S-3).

Table III shows the distribution of fully thiolated and mono, di and tri-oxidized by-products as a function of the position of the phosphorothiolated region of the  
25 oligonucleotide. Four thymidyl pentadodecamers were synthesized using 0.02 M I<sub>2</sub> as the oxidant for 15 nucleotides and a thiolating agent for nine nucleotides.

TABLE 3

| 30 | Ts   | [I <sub>2</sub> ] | S   | S-1 | S-2  | S-3 |
|----|--|-------------------|-----|-----|------|-----|
|    | 5'-9D <sub>s</sub> 15D <sub>o</sub> D03'                 | 0.02M             | 96% | 4%  | -    | -   |
|    | 5'-1D <sub>o</sub> 9D <sub>s</sub> 14D <sub>o</sub> D-3' | 0.02M             | 85% | 15% | -    | -   |
|    | 5'-8D <sub>o</sub> 9D <sub>s</sub> 7D <sub>o</sub> D-3'  | 0.02M             | 41% | 46% | 12.5 | 0.5 |
|    | 5'-15D <sub>o</sub> 9D <sub>s</sub> D-3'                 | 0.02M             | 32% | 43% | 20%  | 5%  |
| 35 | 5'-15D <sub>o</sub> 9D <sub>s</sub> D-3'                 | 0.001M            | 78% | 14% | 8%   | -   |

The results demonstrated that 96% of the 5'S oligonucleotides are fully thiolated, which percentage steadily decreases as the phosphorothioate region is exposed to more frequent oxidation reactions. When the oxidant concentration was reduced to 0.001M, 78% fully thiolated 3'S 25-T oligonucleotides and about 60% of oligonucleotides having the sequence of the SEQ ID NO: 1 were synthesized.

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